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(54) Title: METHOD TO REGENERATE PLANTS AND USES THEREOF TO MULTIPLY AND/OR TRANSFORM PLANTS

(57) Abstract: A method to produce vegetal cellular bulks with high regeneration capability comprising both a chemical and a mechanical treatment and plant cell bulks obtained therefrom is described. The method of the invention is used advantageously to produce meristematic platforms with high regeneration capability, for the vegetative multiplication of a plant and achievement of a gene transformed plant.

METHOD TO REGENERATE PLANTS AND USES THEREOF TO MULTIPLY AND/OR TRANSFORM PLANTS

The present invention relates to a method for the plant regeneration by induction of adventitious meristems in explants, as well as to applications thereof to multiply and transform plant species.

More particularly the invention relates to a method to regenerate by caulogenesis starting from meristematic bulks, *in vitro* induced in somatic tissue explants, by a breakage of the apical dominance in proliferating shoots and by a hyper-cytokininic stimulus, which determines an hyperplasic and hypertrophic development of the shoot base and then a cellular bulk with a complex cellular and tissue structure. The cellular bulk is characterised by the predominant presence of cells with an high caulogenetic regenerative competence.

The method is applicable for vegetative propagation and multiplication as well as for the genetic manipulation (mediated or direct) of various plant species.

The method allows an efficient regeneration of shoots and therefore of plants, from vegetal tissues. The method can be widely utilised during plant genetic multiplication and transformation.

The regeneration occurs from a "platform" tissue characterised by an high number of cells with high competence for the formation of adventitious meristems (Meristematic Platform). The methodology is of great importance due to the possibility to increase the efficiency and/or to extend both *in vitro* multiplication and genetic transformation techniques to many species of agronomic interest. Therefore the method, increasing the efficiency of multiplication methods, in addition to its use for genetic transformation, allows to implement the application of the vegetal biotechnologies also to plants which are not easily manipulated, also providing cost reduction and standardisation of production techniques.

Background of the Invention

In vitro culturing techniques and regeneration

The *in vitro* cultures consist of the growth of cells, tissues or organs, isolated from a parent plant on artificial substrates. They are used both for the vegetative propagation and genetic vegetal improvement. They allow a rapid multiplication also in very narrow spaces, starting from minimal amounts of starting material, independently from seasonal cycles. Due to

the potential of the genetic variability generation and selection, in addition to the combination with genetic manipulation techniques, the *in vitro* cultures are an effective tool supporting the conventional techniques for the genetic improvement. These advantages are yet accompanied by drawbacks, as need of specialised workers, higher costs, potential occurrence of undesirable characters in the seedlings and difficulties in the soil adaptation.

The conventional micropropagation technique is based on the proliferation of apical and axillary buds. Direct or indirect differentiation of adventitious buds and/or somatic embryos from de-differentiated tissue allows possible alternatives but, due to the high genetic variability often induced thereby, it results in a difficult practical application in the propagation systems.

Meristematic tissue cultures

Meristematic tissues are undifferentiated tissues causing the growing process of the plant. Meristematic cells are actively self-divided generating both other meristematic and differentiated cells. Meristems can be derived directly from zygote or adult cells regressing to de-differentiated state; they are named "primary" (for example vegetative and root tips) and "secondary" (for example cambium, adventitious meristems and meristemoids) in the first and second case, respectively. The propagation, conventional and *in vitro*, allows to produce, except in the presence of mutations, a clonal population (group of genetically identical individuals)

Propagation Techniques

The more diffused method for the propagation is shoots culture. The propagation occurs by the generation of axillary shoots, isolated and used as explants for subsequent sub-cultures. As primary explants usually lateral 5-10 mm long apexes or buds are used.

The culture of the terminal ends of the meristematic apexes is named meristem culture. This technique is usually used to remove viruses form the plants, often in combination with heat treatment. Explants consists of small portions (0,2-1 mm long) of apical meristems, with one or two leaf primordia.

Nodus culture is a technique derived from shoot culture, more recently provided (Wang, 1977), wherein the stems are localised horizontally on solid substrate.The propagation, like in the shoot culture, occurs by axillary buds.

Major steps of the micropropagation were defined by Murashige (1974). Debergh and Maene (1981) proposed to add 0 stage, comprising the steps before the explant.

Step 0: Selection of the parent plant and preparation.

Step 1: Start of the culture.

Step 2: Multiplication.

Step 3: Elongation and induction of the root development.

Step 4: Adaptation.

The passage from step 3 to 4 is critical because the seedlings are adapted to a sterile environment characterised by high humidity, low light intensity and supported by external carbon source (for example sucrose). Therefore it is required to control, after the transplant in a yet sterile soil, the light and humidity conditions until the plants re-acquired the structural and functional autonomy. Adaptation yields depend on the type and quality of the material derived from the previous steps.

Morphogenesis and Differentiation

The generation process of a new organ from a vegetal tissue is named organogenesis or morphogenesis. The organs developing from somatic tissues are named adventitious.

In the plants the potential to produce organs or even a complete plant, is owned not only by the meristematic but also by the somatic tissue cells. However not all the plant cells maintain morphogenetic or organogenetic capabilities: the more specialised plant cells, as vascular tissue and sclerenchyma ones, do not modify their differentiated state.

De-differentiation and Callogenesis

The cell and tissue transition from a differentiated to undifferentiated state is named de-differentiation or callogenesis and it can be induced *in vivo* or *in vitro* following chemical, physical or biological stimuli.

Vegetal tissues can growth in an organised way, wherein the cells maintain a well defined structure or in an unorganised way (callus). The organised tissue is constituted of differentiated cells (morphologically and

functionally organised), while the unorganised tissue contains a minimal amount of differentiated cells.

Callus is an amorphous tissue generated when cells are divided disorderly. *In vivo* it can be induced in a plant from produced injury, in the presence of microorganism or stress conditions. During the formation of callus, the cellular metabolism passes from a differentiated and quiescent to a meristematic, not specialised and actively dividing state. The *in vitro* induction of callus takes advantage of the same phenomenon which can be amplified or inhibited by the application of chemical and physical factors. The *in vitro* production of callus is increased by carrying out further cuts on explants before their transfer onto induction substrates and by the combination of growth regulators added to the substrate the light and temperature conditions being adjusted. Three are the major factors inducing the callus: 1) explant type, 2) selection of the substrate and culturing conditions and 3) separation of the callus from the explant and culturing maintenance thereof (Constabel, 1984).

Morphogenetic Processes

De-differentiated cells able to generate new organs or plants, as a response to specified stimuli, are named competent. Competent cells can be induced in a determined state wherein they tend to follow a precise, genetically determined, development path; this path can go on cellular differentiation or morphogenesis (Christianson, 1987). The assumption of the determined state can take advantage of the presence of certain growth regulators.

Major morphogenetic processes from somatic tissue, both differentiated and undifferentiated, can lead to the formation of unipolar or bipolar structures. Within the first class are caulogenesis and rhizogenesis, which include the formation of adventitious shoots and roots, respectively; while somatic embryogenesis leads to the formation of somatic embryos having a bipolar development.

Morphogenesis can be direct or indirect, depending on its occurrence in the presence or absence of an undifferentiated proliferating tissue (Hicks, 1980). Most of studied vegetal species is able to generate adventitious roots and buds from explants of various plant tissues and organs, while the formation of adventitious embryos is very less usual.

Caulogenesis and rhizogenesis

Direct caulogenesis consists of the formation of shoots from somatic tissue, while in the indirect one the shoots are formed from dedifferentiated tissue. In the direct caulogenesis about 48 hours following the transfer of a tissue explant onto the culture substrate initiate mitosis which can originate the meristems of the shoots. In turn meristems can generate primordia, whose disposition appears to be random, tendentiously equidistant.

Absence of chimera indicates that the shoots are generated from an epidermis individual cell or few daughter cells, anyway deriving from a single cell (Broertjies and Van Harten, 1978; Broertjes and Keen, 1980). According to other (Norris and Smith, 1981) the beginning of the shoot meristems includes the association and possible inclusion of cells situated below the epidermis. Ability to form adventitious shoots is anyway dependent on plant genotype and often tissue type of the explant too.

Indirect formation of shoots follows a path similar to the direct one: the rapid division of cells leads to the formation of meristematic or meristimoid centers (Torrey, 1966) which can develop in primordia (Bonnet and Torrey, 1966) and successively shoots. Also in this case it is very probable the unicellular origin of the callus structures (Thorpe, 1982), but cells close to the initial one are often induced to the division and incorporation into the new structure (Chlyah, 1974; Smith and Thorpe, 1975).

Media promoting the callus development usually do not promote morphogenesis too. However in certain cases, if callus is maintained on the same substrate over long time, organogenesis phenomena are observed. In other cases, in order to induce caulogenesis, it is required the transfer of the callus onto suitable substrates. Cells which will form organs derive from the re-setting of undifferentiated cells or cells maintaining a morphogenetic potential already present in the tissue explant. Except some cases the formation of shoots is promoted in recently isolated tissues and the potential is decreasing with the sub-culture number. Sometime callus lines isolated from the same explant can result in different regenerative responses. This behaviour could result from the fact that primary callus consists of competent and not competent cells or tissues (Street, 1979). Morphogenetic and not morphogenetic callus lines

can maintain their own characteristics over years. Sometimes it is possible, by using particular chemical or physical factors, to induce shoots or roots from apparently not regenerative calli, but often these attempts are unsuccessful. Plants with high regenerative capability and showing low genetic variability can be propagated by adventitious more than axillary shoots.

Callus culture, along with the subsequent seedling regeneration, can be used if the object of the culture is not the propagation but the induction of a new variability to be used for the genetic improvement.

Rhizogenesis is the process by which the roots are formed and, as caulogenesis, can occur directly, from somatic tissue or indirectly through an unorganised step. Firstly rhizogenesis proceeds, till to the primordium induction, in the same manner as caulogenesis. Successively primordia can be differentiated in shoots or roots, as a response to stimuli of different typei (Halperlin, 1069). High levels of auxin usually stimulate the rhizogenesis. Generally in vitro rhizogenesis occurs much more easily than caulogenesis.

Direct caulogenesis often is used for the propagation of various decorative and crop plants. In the past some decorative plants like *Fresia* (Hussey and Hargraves, 1974) and *Pelargonium* (Holdgate, 1977) were propagated by indirect formation of adventitious shoots. However currently it is generally consolidated that this process involves a too high variability to maintain the commercial characteristics of the plant.

Adventitious roots and shoots can associated to produce a plant according to different techniques: 1) the caulogenesis is followed by formation of adventitious roots (Skoog, 1944); 2) the adventitious buds are produced on adventitious roots (Earle and Torrey, 1965); 3) the independent regeneration of roots and buds is followed by their integration in a single axis (Steward *et al.*, 1958; Pilet, 1961; Kato and Takeuchi, 1963).

Somatic Embryogenesis

Somatic embryogenesis can occur directly or indirectly. *In vitro* direct embryogenesis consists of the formation of somatic embryos on tissue explants without involvement of undifferentiated tissue. *In vitro* direct embryogenesis principally was described in gametophyte and sporophyte tissues, in association with gametophyte or derived from

fertilisation thereof (Smith and Krikorian). Practically it occurs only in cells suitable to produce embryos, named pre-embryogenically determined cells (PEDC) and the transfer onto the culture medium has only the function to increase such a process (Sharp *et al.*, 1980; Evans *et al.*, 1981a and Sharp and Evans, 1982).

Female gametophyte of some species, as certain *Citrus* species, is able to generate naturally adventitious somatic embryos. This tendency to have in the same gametophyte more than one embryo, namely one zygotic and several adventitious, is named polyembryony.

Somatic embryos can be formed indirectly when undifferentiated callus cells are induced to the embryogenetic determination (Sharp *et al.*, 1980). These cells are named "induced embryogenetic determined cells" (IEDC). Although the embryos are difficult to be observed, they can be distinguished from adventitious shoots in that, differently from these, they are bipolar, comprising both an apical and a radical pole, an axis of the shoot and cotyledons, do not have vascular connections with adjacent parental tissues. Embryos (both zygotic and somatic) are in fact new individuals deriving from single cells and not having vascular connections with parental tissues (Haccius, 1978).

Somatic embryos can develop from a single cell, directly from somatic tissue, on callus or cellular suspensions (Backs-Husemann and Reinert, 1970; Nomura and Komamine, 1986a; Miura and Tabata, 1986) and from protoplasts (Miura and Tabata, 1986). Embryogenetically determined cell can be subjected to a periclinal division forming a cytoplasm rich terminal cell, which will generate embryo, and a large and vacuolated basal cell which in turn can divide a few times generating a suspensor. If the basal cell divides according to anticlinal planes or randomly, can generate pro-embryonal cellular complexes or anomalous embryos with multi-serial suspensor (Trigiano *et al.*, 1989).

In the indirect embryogenesis most of the regenerated plants is genetically normal, higher variability being found in somatic embryos induced in callus cultures or suspension following a long period under not optimal growth conditions (Orton, 1985). Propagation by indirect somatic embryogenesis is applied to few species, usually monocotyledons like cereals and palms, wherein the propagation by the shoots culture or caulogenesis is ineffective. In some cases direct embryogenesis was

effectively used for the micropropagation, as for *Helianthus annuus* L. (Pelissier *et al.*, 1990) and *Juglans regia* L.(McGranahan *et al.*, 1988b).

Possibility to obtain effective differentiation protocols for the various iinteresting species is usually determined by the study and acknowledgement of the genotype response (plant, cell, tissue) to various factors possibly affecting the regulation of the various steps of the morphogenetic process (induction, determination and development).

Usually in this process are involved the following factors:

- growth regulators (phytohormones, stress inductors, oligosaccharides, etc.);
- physical factors (light and temperature);
- regeneration methods.

Within this scope therefore studies about the relationship among these factors are particularly important in order to define effective methods for the regeneration of plants, which are also characterised by high genetic stability, mainly when the application relates to the multiplication or genetic manipulation thereof.

Variability of the morphogenetic processes

Plants regenerated by embryogenesis are usually genetically homogenous, while in some cases, when regeneration by caulogenesis or rhizogenesis is used, it is possible to produce chimeras. The reason of such a behaviour is based on the fact that the adventitious organs can likely have a multicellular origin (Norris and Smith, 1981), while the embryos usually have a unicellular origin.

From above it is apparent the advantage of providing vegetative propagation systems based on the production of cells with high regenerative competence without a long induction period and de-differentiating cultures as a guarantee of high genetic stability. It is further advantageous the insertion of genes in individual cells and the production by regeneration of homogeneously transformed plants for the expression of one or more heterologous genes.

Genetic Transformation

Transformation is a process by which exogenous DNA is introduced into a vegetal cell. In the middle eighty first transgenic plant was produced

from *Nicotiana plumbaginifolia* by the *Agrobacterium* mediated technique (Horsch *et al.*, 1984; DeBlock *et al.*, 1984). Thereafter a great number of species was engineered using different methodologies (Gasser and Fraley, 1989). One of such techniques uses the transformation of vegetal cells from a tissue explant (protoplasts). The production of transgenic plants depends essentially on the introduction frequency of the interest gene and the ability of the transformed cells to regenerate the whole plant, shoots or roots. Both the aspects can represent limiting factors for the fruit plants.

Evidence of the occurred transformation is represented by the expression of introduced DNA in the vegetal cells: for this reason into the transforming vector also a marker gene is inserted which, when expressed, provides the plant with a characteristic phenotype.

To obtain transgenic plants it is required: a) to isolate the interest gene; b) to have a vector system allowing the insertion thereof into the plant; c) to have a good transformation technique; d) to have a good regeneration technique; e) to have a good selection technique.

Genetic transformation techniques

Major transformation techniques can be indirect, when they take advantage of the intrinsic ability of the pathogenic bacterial and viral agents to transfer genes, or direct, wherein exogenous DNA is directly inserted into the cell, without any interaction with pathogenic agent.

Indirect methods use microbial expression vectors. Gene transfer into the vegetal cell is facilitated by the naturally occrring gene transfer system of *Agrobacterium tumefaciens* (Gheysen *et al.*, 1985; Fraley *et al.*, 1986; Lichtenstein and Fuller, 1987; Zambryski *et al.*, 1989; Schuerman and Dandekar, 1991). Also DNA (Gronenborg and Matzeit, 1989) and RNA (French *et al.*, 1986) viruses were modified in order to insert an exogenous gene replacing part of the viral genome, thus generating a viral defective particle able to carry out gene transfer.

The *Agrobacterium* mediated transformation technique was the first tool for the transformation of many dicotyledonae. It is certainly preferred method for all the vegetal species for which T-DNA transfer is possible due to relative transfer easiness and precision in undamaged and able to regenerate explants explants.

However many vegetal species, among which economically important cereals like rice, maize and wheat, are not easily transformed by conventional indirect method. Therefore alternative systems were provided wherein DNA insertion into the host cell occurs as free DNA followed by the random integration into the genome. Among these the biolistic method represent a mechanical method for the introduction of DNA into most of the vegetal species. It is advantageous in all the situations wherein the *Agrobacterium* mediated transformation and free transfer into protoplasts are not possible. The technique is based on the acceleration of heavy particles with some micrometer diameter, principally gold or tungsten, which can penetrate through the cellular wall and plasmatic membrane of undamaged vegetal cells, thus delivering the genetic matter. Because the small holes through the wall and plasmalemma quickly self-close, the produced injuries are temporary and do not compromise irreversibly the cell integrity.

The first transgenic plant, simultaneously obtained by *Agrobacterium* (Hincee *et al.*, 1988) and biolistic method (McCabe *et al.*, 1988), was soy-bean. The most interesting result was the production of fertile transgenic maize plants (Fromm *et al.*, 1990; Gordon-Kamm *et al.*, 1990).

Selection Systems

Many genes encoding for antibiotics or herbicides can be used as selection markers, usually required for an efficient production of transgenic plants. Selection agents are different each other relating to the toxicity grade against the plant, which depends on the size and development step of the cell o tissues. In the presence of the selection agent indifferentiated cells respond differently to the explants of organs as leaves and cotyledons. In addition the susceptibility to the various agents depends on the species. Therefore for each transformation and regeneration protocol it is required to carry out the precise determination of the concentration of the selection agent which allows on the hand the growth and development of the transformed cells and on the other hand inhibits the proliferation of undifferentiated cells. Often herbicides are more toxic than antibiotics against vegetal tissues such that their use is carried out only successively, i.e. when it is certain that the transformed cells produce enzymes in such amount to induce resistance (Donn *et al.*, 1990).

For many herbaceous species, usually propagated by gametes, are provided effective efficacious protocols for regeneration, also by somatic embryogenesis, which afford effective multiplication and transformation results. For some of herbaceous and mainly many of the arboreal species there are yet remarkable difficulties in developing efficacious regeneration protocols suitable for genetic multiplication and transformation. In many cases there are specific problems also for the application of standard transformation protocols.

The method of the invention aims to overcome these problems, by providing an efficacious genetic transformation protocol also for plants proving recalcitrant to the conventional techniques.

Prior art techniques prove to have an effective application only for species having a cellular structure of the various somatic tissues characterised by high cellular competence for organogenesis and somatic embryogenesis processes. Currently known and/or used regeneration protocols depend on the cellular competence of the interest plant genotype (Genus-Species-Variety) and tissue type (both meristematic and somatic: leaves, stem, stipule, etc.). Many vegetal species having high agronomic interest show an extremely reduced competence to the regeneration of their various cellular typologies. This behaviour, mainly apparent in many woody species, often represents the determining factor in limiting the application of the *in vitro* multiplication technologies and gene manipulation, due to reduced efficiency and difficulty in obtaining the regeneration of interest gene modified plant, respectively.

The method of the invention allows to obtain the formation of tissues characterised by a major cellular component having high competence for the regeneration of adventitious buds. For example, the method allows to rationalise the production process of nurseries because it can be used both for the multiplication and transformation. Thus the costs for the production and implementation of genetic vegetal biotechnological innovations are reduced.

Description of the Invention

The present invention consists of method of *in vitro* culturing plant tissues that, by inducing and culturing meristematic cellular bulks, allows to produce cellular layers (platforms) characterised by an high regeneration efficiency, via caulogenesis, of new plants.

The basic principle of the method is the induction of growing cellular bulks with high regenerative competence from which, via sectioning, it is possible to obtain cellular layers (platforms), again with high regenerative competence. The choice of the initial concentration of the growth regulators is defined on the base of the indications from individual species during the *in vitro* standard proliferation step (proliferation of axillary buds).

Availability of *in vitro* proliferating shoots and knowledge of growth regulators (particularly cytokinins) combinations required for the standard proliferation of axillary buds prove to be essential for the induction and production of meristematic bulks). Therefore culture substrates characterised by higher cytokinin concentrations were provided, thereby inducing higher *in vitro* proliferation of axillary buds, which results in the generation of dense cellular bulks consisting of different type meristematic tissues. This evolution usually is reached during 3-4 sequential sub-cultures wherein the initial concentration of cytokinins in the substrate gradually is triplicated. In parallel to this hormonal treatment at each culture all the more developed bud apexes are removed (topping) not touching the base. Therefore the method of the invention to produce meristematic bulks is based on the combined effect of the chemical (hormonal) and mechanical (breakage of the apical dominance: topping) treatment.

The chemical treatment consists of progressive increase of the cytokininis content in the substrate of the proliferating shoots, reaching in few sub-cultures (3-5) an effective concentration of the cytikininis at least three times higher than initial value in the proliferating substrate (1-3).

The mechanical treatment includes, at each transplant of the proliferating shoots, without removing the base, the removal of the apex of the individual shoots (topping) originating from axillary buds. This treatment promotes a strong growth of the base which more and more is characterised by an increasing presence of various meristematic centres.

Thus the not removed base of the shoots becomes more and more developed and hypertrophic till to the formation of a bulk characterised by an parenchymatic base with cells having high competence for the differentiation of adventitious buds. Histologically these bulks are characterised by a more internal part of dense tissue, made of the shoot base co-growth with the buds. Cross-sections of the bulks show the

simultaneous presence of primary and secondary meristems and pro-meristematic primordia immersed in highly vascularized parenchyma. Particularly it is apparent high formation, also in very limited tissue portions, of primary meristems at different evolution steps: from

parenchymatic cells (callus) to complete primary meristem (adventitious bud) comprising all the intermediates steps (nodules, meristematic primordium). These cellular bulks therefore prove to be with high regeneration competence which can be maintained during successive sub-culture on substrates characterised by the cytokininis concentrations reached in the last step of the bulk induction. The essential characteristic of the produced bulks is that from their fragmentation, following three or four culturing weeks, identical formations are obtained. In fact these meristematic bulks can be maintained in culture and multiplied by sectioning (about 2 mm thick homogenous sections) resulting in new meristematic platforms characterised by the same regenerative efficiency.

This "meristematic bulk-platform" cycle prove to be constant for the various analysed species and usable both for the vegetative multiplication (cloning) and genetic transfer.

Application 1 – Vegetative Multiplication

Effective induction and maintenance of meristematic bulks (MB) and high regenerative efficiency of new adventitious shoots of meristematic platforms represent an effective multiplication method resulting in simplified techniques for the in vitro manipulation of the vegetal matter (simple sectioning of the bulks) and the achievement of explants maintaining yet high regenerative efficiency.

The method is suitable to produce large amounts of these cellular bulks (both on liquid and solid substrates), easily to be sectioned and usable for the production and differentiation of new shoots and plants in great number. Thus formed meristematic bulks can be used for the production, via regular sectioning, of cellular platforms (about 2 cm thick and 1 cm long and wide sections) which maintain the characteristic of meristematic-vascularized bulks with high competence to determine the regeneration of new adventitious shoots (caulogenesis).

The application of the method for the vegetal propagation is demonstrated by effective and stable proliferation of the bulks (via

regeneration) and effective regeneration obtained from cellular platforms by mechanical fragmentation thereof. High efficiency of this technique can be further increased by potential automation-robotization of some steps (bioreactor-grinder).

Application 2 – Genetic Transformation

Cellular platforms obtained by the method of the invention are advantageously applied for the genetic transfer both by *Agrobacterium tumefaciens* mediation and direct techniques. In this case the combined effect of the injury and high frequency of regeneration competent cells promote high efficiency also for the transformation.

Characteristics of the cells constituting meristematic bulks and sectioning thereof, carried out during the regeneration-multiplication step, assist to create optimum conditions to promote genetic transformation events. Already formed meristems, not competent for the transformation, develop shoots which will be removed over a few selection cycles

Particularly experiments using *Agrobacterium tumefaciens* EHA 105 strain containing pTi EHA105 unarmed plasmid (Hood *et al.*, 1993) and various vectors, particularly the binary vector containing neomycin photo transferase marker gene (nptII), which confers kanamycin resistance, under the control of nopaline synthetase promoter (Pnos), are carried out.

First operation to be carried out in the preparation of the transformation explants is the removal of all the outer surface portion, exposing inner tissue till to the formation of about 1 cm small cube made of clear and dense tissue. Following the small cube is sectioned forming cellular platforms showing high regenerative efficiency and large injury surface, considered necessary to promote the efficient transformation. In fact, mainly referring to *Agrobacterium* mediated transformation, the infection and transformation capability is affected by the activity of the compounds released from the surface of the injured cells during the preparation of the sections.

For the growth and infection of the bacterial strains standard techniques are used.

The infection, further to growth of bacteria, is carried out according to the conventional PM infection method, preferably including also a treatment with acetosyringone and syringaldehyde (25 µl), which are

organic compounds known to activate the expression of *vir* genes and, therefore, to mobilise the DNA of the binary plasmid.

At the end of infection the explants are dehydrated using 3MM sterile absorbent paper and transferred into Petri dishes containing the regeneration substrate characterised by hormonal concentration as defined for the PM regeneration. Anyway the transformation occurs for an extremely reduced number of cells and the following selection step is extremely important to discriminate stable and homogeneous transformation events. To this object a protocol of continuous regeneration in selecting medium, based on various passages of PM on substrates added with same concentrations of growth regulators and increasing doses of the selecting factor (kanamycin), from a minimum level of 25 mg/l to a final concentration (after 4 sub-cultures) from 50 to 100 mg/l, depending on the kanamycin sensitivity of the species, is started.

First shoots developing from PM usually originate from already formed meristems, therefore probably without stabile transgenic origin. However it is extremely important to remove no part of tissue or shoots during the initial phase of the selection, in order to maintain also new partially modified shoots (transgenic chimeras). Very often in these cases only few cells of the meristem or section thereof are transformed. In this way chimeric individuals are formed. By maintaining the selective pressure of kanamycin the probability of isolating the chimera with subsequent advantage for transgenic cells increases. In fact also for in vitro cultures it is difficult to equilibrate the chimeras which usually result in an advantage to one of two genotypes.

This is explained by a different response to the in vitro culture conditions of two genotypes. With a transgenic-not transgenic chimera the selective pressure exerted by kanamycin results in selective advantage of transgenic cells.

A moderate selection during proliferation and subsequent radication with kanamycin (in radication it is possible to obtain a better selection) are preferred for easy rooting species. It is extremely important to proceed by a continuous regeneration over quite long periods and frequent sub-cultures. Continuous regeneration means to maintain, after the first regeneration, continuously the explants in regeneration and selection substrate. In fact it is believed that the initial regeneration events usually

are related to already formed and therefore rarely transgenic meristems. In a following step, the vegetal matter being maintained in a regeneration-selection substrate, the probability to increase the frequency of transformed cells, whose subsequent differentiation promotes the regeneration and development of permanently and homogeneously genetically modified individuals, is higher.

Continuous selective regeneration from PM obtained regenerations results in an amplification of differentiated tissues with genetically manipulated cells and consequent stabilisation of chimeras and increase of the transgenic frequency. To verify the transformation homogeneity of isolated shoots a rooting final step is carried out again in the presence of kanamycin. In fact the generation of roots in the presence of kanamycin corresponds, very probably, to an occurred transformation.

The present invention will be described below by way of specific embodiments to be considered illustrative but not limiting, wherein reference will be made to the following figures:

Figure 1. Southern Blot analysis on 0,7 % agarose gel in 0,5 X TBE of DNA of *Defh9-iaaM* transgenic, vine, cv "Thompson". Column 1, HindIII digested clone 4 DNA. Column 2, HindIII/EcoRI digested clone 4 DNA.

Figure 2. Southern Blot analysis of DNA of two *DefH9-iaaM* transgenic, vine, cv Silcora (I.G. 235023). Column 1, HindIII digested clone 29 DNA. Column 2, HindIII/EcoRI digested clone 29 DNA. Column 3, HindIII digested clone 35 DNA. Column 4, HindIII/EcoRI digested clone 35 DNA. Column 5, HindIII digested control DNA. Column 6, HindIII/EcoRI digested control DNA.

Vegetative Multiplication

The described method for the regeneration by the formation of a "platform" tissue characterised by very numerous cells with high competence state for the formation of adventitious meristems (Meristematic Platform) has been tested and developed for a great number of species of agronomic and forest interest.

Description of the method applicable for dessert grapes (*Vitis vinifera*; cvs Thompson Seedless and Silcora (I.G. 235023)

Meristematic bulks induced using proliferating shoots, are *in vitro* stabilised in 500 cc "Fidenza" pots in A substrate containing: macroelements listed in Table 1; microelements and vitamins according to

Murashige and Skoog (1962); and 0,8 mg/l 6-Benzyl adenine hormone; 3 % sucrose; 0,7 % Agar.

Table 1

Macroelements	(mg/l)
KNO_3	1050
NH_4NO_3	400
KH_2PO_4	200
$MgSO_4\ 7H_2O$	400
$CaNO_3$	750
NaH_2PO_4	200

Meristematic bulks are subjected, at each sub-culture, to a severe topping to stimulate a more and more increasing proliferation at the base of the cluster, according to the scheme showed in Table 2.

Table 2

First sub-culture	Second Sub-culture	Third sub-culture	Fourth sub-culture
Severe topping without removal of the cluster base	Severe topping without removal of the cluster base	Severe topping without removal of the cluster base	Production and maintenance of Meristematic Bulks
Day 0	Day 30	Day 60	Day 90
BAP 1 mg/l	BAP 2 mg/l	BAP 3 mg/l	BAP 3 mg/l
NAA 0,01 mg/l	NAA 0,01 mg/l	NAA 0,01 mg/l	NAA 0,01 mg/l

Shoots are stimulated at the same time with hormonal and mechanical stimuli together with severe topping. In this way the shoots become more and more large and hypertrophic, till to obtain bulks wherein buds and shoots are co-growth in a dense and callus and meristematic primordia rich bulk. Histologically the bulks are characterised by more internal part of dense tissue consisting of the shoot base co-growth with the buds. In the cross-section many bulks of primary and secondary meristems and pro-meristematic primordia immersed in a richly vascularised parenchyma are apparent.

Meristematic bulks easily can be propagated by manual or mechanical fragmentation. From these further bulks on the same substrate, containing 3 mg/l BAP for the induction, can be obtained.

By removing the external part of the bulks, rich of already formed meristems and buds, sections, of about of 1 cm^2 surface and 2 mm thickness, can be prepared to constitute MPs usable for the multiplication and transformation.

Results of the regeneration by multiplication process

The described method allows to produce the meristematic platforms with high regeneration capability, resulting in higher production efficiency of shoots than proliferating rate usually found during a conventional *in vitro* proliferation step from axillary buds and also than regeneration rate from somatic tissue (usually leaf and stalk).

In Table 3 the proliferation rates obtained on overage for the two vine varieties by applying the PM regeneration and conventional proliferation and regeneration methods from axillary buds and somatic tissues, respectively, are compared.

Table 3

Proliferation rate from axillary buds	Regeneration rate from foliar or other somatic tissues	PM regeneration rate
3-5	0,1-0,5	20-30

Use of the method for other agronomically interesting species

Analogously the method is used for a great number for other species, for which was proved the possibility of obtaining the formation of the meristematic bulk. Particularly good results of the PM regeneration efficiency for the species listed in Table 4 were obtained.

Table 4

Temperate fruit	Sub-tropical fruit	Horticultural	Officinal forest shrubby
Peach	Almond	Artichoke	Eucaliptus
Pear	Orange	Been	Walnut
Apple			
Kiwi			
Strawberry			

In Table 5 the proliferation rates obtained for the various tested species by applying the PM regeneration and conventional proliferation

and regeneration methods from axillary buds and somatic tissues, respectively, are compared.

Table 5

Species	Proliferation rate from axillary buds	Proliferation rate from foliar or other somatic tissues	PM regeneration rate
Peach	2-4	0,01-0,1	20-30
Pear	2-4	0,01-0,5	20-30
Apple	3-5	0,1-0,8	20-30
Kiwi	2-3	0,1-0,8	10-15
Strawberry	5-8	0,1-0,8	15-25
Almond	3-5	0,01-0,1	20-25
Orange	3-5	0,01-0,1	10-15
Artichoke	3-5	0-0,0,1	20-25
Bean	3-5	0-0,01	15-20
Eucaliptus	4-6	0,01-0,1	20-30
Walnut	2-4	0-0,2	20-30

Gene Transfer

Using the described PM regeneration, Agrobacterium mediated gene transfer and selection methods, genetically modified shoots were obtained with very high frequency (see Table 6 below).

Description of the method for the vine

Meristematic platforms, after the infection and co-culturing period with *Agrobacterium*, were placed on regeneration substrate consisting of same substrate as used in the third sub-culture during the induction step added with BAP 3 mg/l, cefotaxime 50 mg/l and kanamycin 50 mg/l. This step is important because the transformation occurs for a reduced number of cells and the regeneration, under the selective pressure of kanamycin, gives a selection advantage to the cells and transgenic shoots at each regeneration cycle.

The procedure, repeated for various regeneration cycles, results in the amplification of the transformation events producing a redundancy of transgenic individual copies, but also in increase of the chimera stabilisation and transgenic frequency. The process involves the presence of "escapes" in great number, i.e. apparently transgenic shoots which

actually avoided casually the selection agent effect, requiring therefore a severe final selection.

All the matter is placed to radication on a substrate with these components: macroelements (Quoirin and Lepoivre medium, 1977); microelements and vitamins (Murashige and Skoog medium, 1962); Hormones: IBA 1 mg/land IAA 1 mg/l; sucrose 3 %; agar 0,7 %, kanamycin 50 mg/l.

Molecular analysis

The confirmation of transegenic origin of kanamycin rooted shoots is carried out for two vine varieties by Southern Blot analysis.

Figure 1 shows Southern Blot analysis of *Defh9-iaaM* transgene transgenic cv "Thompson" vine DNA. The probe used, homologous to nptII gene, is of 544 bp, produced by PCR using following primers:

5'CAGAGTCCCGCTCAGAAGAACTCGTCA3' and
5'GGAAGGGACTGGCTGCTATTGGGCGAA3'

The plant contains 4 copies of T-DNA inserted into the genoma. Only one of four fragments, about 8,6, 7,5 and 2,8 kb, is cut by EcoRI, as deduced from the disappearance of the heaviest fragment by double digestion and concurrent detection of 2,4 Kb fragment. Because the distance from HindIII site, present in T-DNA at nptII gene 5', to T-DNA right border (RB) is about 2 kb, EcoRI site is present in genomic DNA of the vine transgenic plant at 400 bp from the insertion site.

Figure 2 shows Southern Blot analysis of *Defh9-iaaM* transgene transgenic cv Silcora (I.G. 235023) two vine DNA. The probe used, homologous to *iaaM* gene encoding region, is of 589 bp, produced by PCR using following primers:

5'AACAAGCTTCCCACCACCATCCAG3' and
5'GCATGCTCTTTTCACCCGTATTAG3'

Clone 29 contains 3 copies of T-DNA inserted into the genoma, as deduced from the presence of three bands of about 1,2, 3,5 and 6 kb, produced by HindIII enzyme digestion and homologous to the probe obtained from *Defh9-iaaM* gene promoter. 35 plant contains only one T-DNA copy, as deduced from the detection of a single band of about 2,1 Kb. In both cases the HinfIII and EcoRI double digestion produce an about 0,8 Kb band.

Table 6

Stable transformation events from 100 *Agrobacterium* infected explants

Species	Regeneration from leaf	Somatic embryogenesis	PM regeneration
Vine	0	2	10

1.

Above described regeneration method already prove to be very efficient, but yet can allow to reach further advantages in the development of new vegetative propagation techniques when adjusted for automated culturing systems (bioreactors). The fragmentation of meristematic bulks results in a continuos tissue growth associated with new meristems differentiation.

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CLAIMS

1. Method to produce cellular vegetal bulks with an high regeneration capability comprising a chemical and a mechanical treatment, wherein the chemical treatment includes:

a) culturing *in vitro* proliferating shoots on a first nutrition substrate comprising cytokinins till to the production of shoots to be transplanted;

b) transplanting the shoots obtained according to a) on a second nutrition substrate comprising cytokinins at a concentration higher than the first substrate till to the production of shoots to be transplanted;

c) if necessary, transplanting the shoots obtained according to b) on a third nutrition substrate comprising cytokinins at a concentration higher than the second substrate till to the production of shoots to be transplanted;

d) if necessary, transplanting the shoots obtained according to c) on a fourth nutrition substrate comprising cytokinins at a concentration higher than the third substrate till to the production of shoots to be transplanted;

and wherein the mechanical treatment essentially consists of the steps of:

e) removing from the shoots to be transplanted at each of the steps from a) to d) the apex without the removal of the base.

f) if necessary, *in vitro* culturing thus obtained bulks.

2. Method to produce cellular vegetal bulks with an high regeneration capability according to claim 1 wherein cytokinins comprise BAP.

3. Cellular vegetal bulks with an high regeneration capability obtainable according to the method of claim 1 or 2.

4. Method to produce meristematic platforms with an high regeneration capability comprising the steps of:

a) obtaining bulks according to claim 1 or 2;

b) sectioning the same in platforms;

c) if necessary, *in vitro* culturing thus obtained meristematic platforms.

5. Method to produce meristematic platforms with an high regeneration capability according to claim 4 wherein thus obtained platforms are about 2 mm thick.

6. Meristematic platforms with an high regeneration capability obtainable according to the method of claim 4 o 5.

7. Method to multiply vegetatively a plant including the steps of:

a) obtaining the meristematic platforms according to claim 4 or 5;

g) inducing in the meristematic platforms the regeneration of adventitious shoots by caulogenesis;

h) obtaining a plant from the adventitious shoots obtained in b).

8. Method to produce a genetically transformed plant by a transgene comprising the steps of:

a) obtaining the meristematic platforms according to claim 4 or 5;

b) transforming the cells of the meristematic platforms using the transgene;

c) maintaining the transformed meristematic platforms in selective medium for at least 3 regeneration cycles, so to obtain an increase of the number of the genetically transformed cells in the platform;

d) regenerating the plant from said platforms maintained in selective medium.

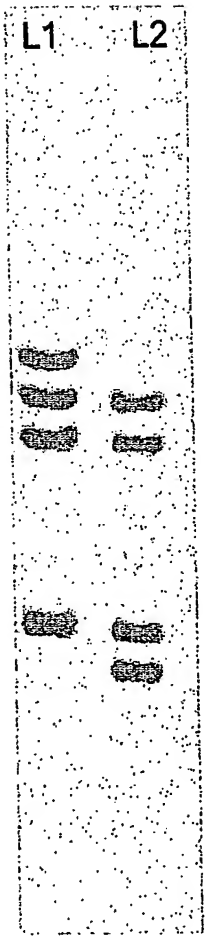

Fig. 1

Fig. 2